Organellar (Na^+, K^+)/H^+ exchanger NHE7 regulates cell adhesion, invasion and anchorage-independent growth of breast cancer MDA-MB-231 cells

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Abstract. Na^+/H^+ exchangers (NHEs) are a group of secondary active antiporters that regulate cellular pH, cell volume and ion homeostasis. In humans, nine isoforms (NHE1-NHE9) were identified and characterized as functional NHEs. While a growing body of evidence indicates that NHE1 generates an acidic tumor environment and thereby contributes to tumor invasion, little is known about the role of other NHE isoforms in tumor progression. NHE7 is a unique member of the NHE gene family that dynamically shuttles between the *trans*-Golgi network, endosomes and the plasma membrane, and regulates the luminal pH of these organelles. Here we show that NHE7-overexpression in breast cancer MDA-MB-231 cells enhances cell overlay, cell-cell adhesion, invasion, anchorage-independent tumor growth and tumor formation *in vivo*. In contrast, NHE1-overexpression enhances tumor invasion, but it has little effect on cell adhesion or anchorage-independent tumor growth. Pathological examinations of the tumor samples derived from NHE7-overexpressing cells showed a similar appearance to aggressive tumors. Together, these results suggest that NHE7 enhances tumor progression. This is the first report to show the involvement of an organellar NHE in oncogenic processes.

Introduction

An acidic microenvironment is a hallmark of malignant tumors, which is facilitated by altered metabolism of tumor cells and the hypoxic tumor microenvironment (1-4). Mounting evidence indicates the involvement of Na^+/H^+ exchangers (NHEs) in tumor migration, invasion and metastasis (5-8). A model has been proposed in which excessive glycolysis in malignant tumors leads to the accumulation of lactate and protons in the cytosol, activates NHEs and leads to extracellular acidification (6,9-11). To date, nine human genes, NHE1-NHE9, have been isolated and their gene products characterized as functional NHEs (12,13) that display different intracellular localization and tissue expression. Based on the capability to complement Na^+/H^+ exchange activity across the plasma membrane in the NHE-deficient mutant cells (14,15), NHEs can be divided into two classes: plasma membrane-type NHEs (NHE1-NHE5) and organelle membrane-type (organellar) NHEs (NHE6-NHE9). Organellar NHEs are evolutionarily highly conserved from the *S. cerevisiae* NHX1 to the metazoan NHE6-NHE9. NHE7 was identified as the first NHE that can transport either Na^+ or K^+ in exchange for H^+ across the organelle membrane (16). A similar cation-non-specific antiporter activity was subsequently shown in human NHE6, NHE8 and NHE9 (17), and in yeast NHX1 (18). Thus, organellar NHEs are believed to regulate acidic organellar pH along the secretory, endocytic and recycling vesicular trafficking pathways (19,20).

Organellar pH levels along the secretory, endocytic and recycling pathways are tightly controlled and any aberrations in such pH environments could accelerate transformation (21-25) and influence multidrug resistance to anticancer drugs (26). Moreover, it has been suggested that changes in the intra-Golgi pH level alter the structure of the Golgi apparatus and affect protein glycosylation (27,28,81), which may facilitate malignant transformation (29-31). However, despite the potential significance of organellar pH and ion homeostasis on tumor progression, the role of organellar NHEs in oncogenic processes remains undefined. We previously identified a group of NHE7-binding proteins in breast cancer MDA-MB-231 cells and showed that these proteins form the NHE7-interactome, a web of protein-protein interaction network (32). Interestingly, cell adhesion molecules, cell-surface receptors and cytoskeletal proteins were identified as components of the NHE7-interactome, which may form a functional network in tumor cells and regulate tumor progression. Here we show that NHE7 influences cell adhesion,

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anchorage-independent growth and tumor formation *in vivo* using breast cancer MDA-MB-231 cells as a model. Elevated expression of NHE1 facilitates tumor invasion but has little effect on cell-overlay phenotype or anchorage-independent growth. We propose that NHE7 acts as a novel regulator of tumor progression.

Materials and methods

Materials. MDA-MB-231 cells were obtained from ATCC (Manassas, VA, USA). All salts, buffers and other chemical reagents were of analytical grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. DMEM/F12 cell culture media, fetal bovine serum (FBS), trypsin, antibiotics and fluorescence-conjugated secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). Mouse monoclonal antibodies against HA, 1D4, NHE1, actin were obtained from Convance (Princeton, NJ, USA), National Cell Culture Center (Minneapolis, MN, USA) BD Biosciences (Mississauga, ON, Canada) and Sigma, respectively. Polyclonal antibodies against NHE7 were raised and characterized as described (32). Plastic tissue culture dishes, plastic plates and glass coverslips were purchased from Sarstedt/ThermoFisher Scientific (London, ON, Canada). Transwell chamber and Matrigel were obtained from BD Biosciences (Mississauga, ON, Canada). Animals were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA).

Cell culture. MDA-MB-231 cells were routinely maintained in DMEM/F12 media containing 10% fetal bovine serum (FBS) at 37˚C in the presence of 5% CO_2 unless otherwise stated. C-terminally HA-tagged or 1D4-tagged cDNA was cloned into the pcDNA3 vector and the NHE expression constructs or the empty vector were transfected in MDA-MB-231 cells by the calcium phosphate method as described (32). Cells stably expressing these constructs were selected in the presence of 400 μg/ml of G418 and single clones were isolated. Stable cell lines were maintained in the presence of 200 μg/ml and only low passage number cells were used for the experiments.

Western blot analysis. Western blot analysis was carried out as previously described with some modifications (32). In brief, cells were lysed in the cell lysis buffer containing 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate plus protease inhibitor cocktails for 30 min on ice. Cellular debris was removed by centrifugation at 16,000 rcf for 30 min at 4˚C. Proteins (5 μg) were resolved on SDS-PAGE gels, transferred to PVDF membranes and probed with the indicated antibodies. The signal was detected by the Immobilon Western kit (Millipore, Billerica, MA, USA).

Saturation density assays. Stable cell lines ($1x10^5$) were plated in 12-well dishes in DMEM/F12 containing 5% FBS. After 5 h, the medium was replaced with low-serum medium containing 0.5% FBS every 24 h for 8 days. For each cell line, the cell numbers were counted in triplicates every 48 h and the mean cell number and the standard deviations were calculated.

Cell-overlay assay. To establish a feeder layer, $2x10^6$ of MDA-MB-231 cells were seeded to fibronectin-precoated coverslips and maintained at 37˚C in the CO_2 incubator until they reached confluency, which typically occurred after 24 h. Sixteen hours before the experiments, culture media were replaced with fresh media. On the following day, $3x10^5$ of each of the transformed cell lines were seeded over the feeder layer for indicated time periods and fixed with 2% paraformaldehyde/PBS and subjected to immunofluorescence microscopy.

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as previously described (33) with some modifications. In brief, the cells were grown on collagen or fibronectin pre-coated glass coverslips, fixed with 2% paraformaldehyde/PBS (pH 7.4) for 15 min, quickly rinsed with PBS and the plasma membrane was permeabilized with 0.1% Triton X-100/PBS for 5 min. After blocking with 2% normal goat serum/PBS for 20 min, the samples were incubated with primary antibodies. After washing with 0.01% Triton X-100/PBS, the samples were incubated with the Alexa-488-conjugated goat anti-rabbit or Alexa-562-conjugated goat anti-mouse antibodies for 45 min. After extensive washing, the coverslips were mounted on a glass slide and analyzed by fluorescence microscopy.

Matrigel Transwell chamber assays. Matrigel was diluted in serum-free media to a final concentration of 5%, 50 μl of the diluted matrigel was applied to the insert of the Transwell chamber and the pre-coated insert was dried at 37˚C for at least 1 h. The pre-coated inserts were incubated with 1.5 ml of DMEM/F12 media containing 5% FBS in a 12-well plate to prevent excessive drying. After trypsinization and a quick rinse, the same number of cells ($5x10^5$ cells) were resuspended in 500 μl DMEM/F12 plus 0.5% FBS and placed in the upper chamber. Cell migration was stimulated by adding 10% FBS containing DMEM/F12 media to the bottom chamber. The cells were incubated for 48 h in the CO_2 incubator at 37˚C to allow cells to migrate through the matrigel-coated membrane toward the lower chamber. In order to measure invasion, the upper face of the membrane was wiped off to remove non-invading cells. Cells that invaded to the bottom face of the membrane were fixed with ice-cold methanol for 20 min and stained with crystal violet. Cells were scored from five randomly chosen fields of the central regions of the membrane using a light microscope under high power magnification (x400).

Soft agar assay. Sea plaque agar was dissolved in distilled H_2O to 5% and autoclaved. After cooling down to 42˚C, the agar was diluted to a final concentration of 0.5% with pre-warmed DMEM/F12 media supplemented with 10% FBS, and poured into a 35-mm plastic culture dish to make the bottom agar layer. Cells (15,000) suspended in 4.0 ml of the liquid media were mixed with 6.0 ml of the 0.5% agar suspension and immediately layered over the hardened agar in triplicates. After incubating for 6 weeks at 37˚C in the humidified CO_2 incubator, colonies were stained with 0.005% crystal violet and observed under light microscopy at x400 magnification. The average number of colonies was calculated from five randomly chosen fields.

Subcutaneous injections of mice. Clonal MDA-MB-231 cells stably expressing NHE7, NHE1 or empty vector ($4x10^6$ in 50 μl DMEM/F12 media) were subcutaneously implanted into 5-6-weeks-old female CD-1 nu/nu mice under anesthesia with

isoflurane. The tumor size of the primary tumor was scored. All animal experiments were conducted according to the guidelines set by the University of British Columbia.

Tissue preparation and immunohistochemistry. Tumor samples extracted from the animals were fixed overnight in 4% paraformaldehyde/PBS and stored in 70% ethanol. For immunohistochemical analysis, samples were further dehydrated and embedded in paraffin. Sections (4 μm) were prepared, stained with Mayer's hematoxylin staining and processed for immunocytochemistry.

Results

Cell-overlay and cell-cell adhesion are facilitated by NHE7. While characterizing MDA-MB-231 cells overexpressing NHE7 (32), we noted that NHE7-overexpressing cells promptly pile up even before reaching confluency. To explore the potential role of NHE7 overexpression in this phenotype, we began to assess NHE protein expression in MDA-MB-231 cell lines stably expressing NHE7 (N7), NHE1 (N1) and pcDNA vector (c-MDA231). Western blot analysis using an antibody against HA- or 1D4-tag showed the specific heterologous expression of NHE1 and NHE7 (Fig. 1). In agreement with the previous studies (34-37), Western blot analysis revealed two bands for NHE1, a higher molecular weight band representing the fully-glycosylated form and a lower molecular weight band corresponding to the core-glycosylated form. NHE7 exhibited a smeary appearance with two predominant bands, likely representing multiple splicing variants and post-translational modifications as previously reported (32). Overexpression of NHE7 did not influence the endogenous NHE1 protein expression levels and heterologously expressed NHE1 did not affect the endogenous NHE7 protein abundance. N1, N7 and c-MDA231 were seeded onto a 12-well plastic culture plate and the cell numbers were scored. After 48 h of incubation, both N1 cells and c-MDA231 cells reached the maximum number of cells (~7x10^5 cells); the cell number started to decline thereafter. In sharp contrast, N7 cells kept growing for up to 144 h and reached 1.8x10^6 cells, a density ~2.5-fold higher than achieved by N1 and c-MDA231 cells (Fig. 2A). We postulated that the altered cell-contact might have been caused by altered cell-cell adhesion and we next examined the cell adhesiveness. MDA-MB-231 cells were grown on coverslips to confluence (bottom-layer cells) and N1 cells or N7 cells were seeded onto the bottom-layer. After incubation at 37˚C for 30-240 min, cells were fixed and NHE-overexpressing cells adhering to the bottom-layer cells were detected by immunofluorescence microscopy using anti-1D4 or HA antibodies. The number of N7 cells adhering to the bottom cell-layer was 3-5-fold higher than the N1 cells (Fig. 2B). When directly plated on coverslips, no appreciable difference was observed in the plating efficiency between the different cell lines (data not shown). These results suggest that NHE7, but not NHE1, influences cell-overlay and cell-cell adhesion.

Tumor cell invasion is facilitated by the overexpression of either NHE1 or NHE7. Cellular/extracellular pH milieus have a substantial impact on tumor invasion and metastasis (7,38,39), and the involvement of NHE1 (9,40-45). Expression of other

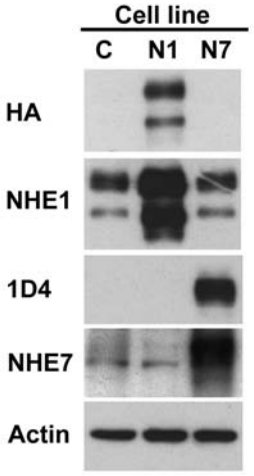

Figure 1. NHE protein expression in MDA-MB-231-based stable cell lines. The same amount of cell lysates (5 μg) was resolved on an SDS-PAGE gel, transferred to a PVDF membrane and the expression of indicated proteins in each sample was analyzed by Western blot analysis. Heterologously expressed HA-tagged NHE1 and 1D4-tagged NHE7 were specifically detected by anti-HA and anti-1D4 antibodies, respectively. Overexpression of NHE1 did not affect endogenous expression of NHE7, and conversely, overexpression of NHE7 did not influence the endogenous NHE1 expression. The actin blot was used as a loading control.

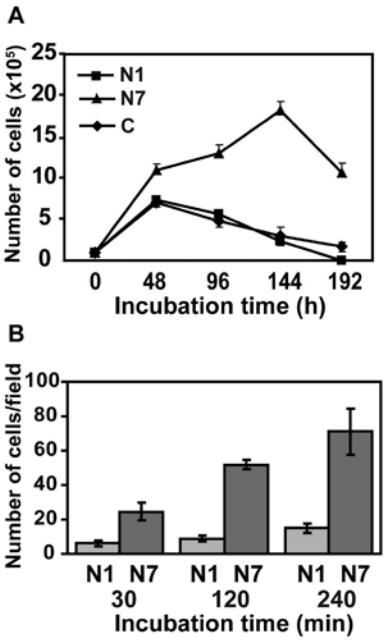

Figure 2. (A) NHE7-overexpressing cells pile up more avidly than NHE1-overexpresing cells or vector control cells: Equal numbers of MDA-MB-231 cells stably expressing HA-tagged NHE1 (N1), 1D4-tagged NHE7 (N7), or the empty vector (c-MDA231, C) were cultured in low-serum media for the indicated time periods and the cell numbers were counted. The data are expressed as mean ± SD. (B) NHE7-overexpression facilitates cell-cell adhesion: Equal numbers of N1 cells and N7 cells were plated onto feeder layer cells and the attached populations were evaluated by immunostaining with anti-HA and anti-1D4 antibodies, respectively. The data are expressed as the mean number of cells per high power field ± SD. A set of representative data of three independent experiments is shown.

NHE isoforms in tumor cells has also been reported (8,46,47), however the role of the elevated activity of organellar NHEs in these processes is currently unknown. In order to investi-

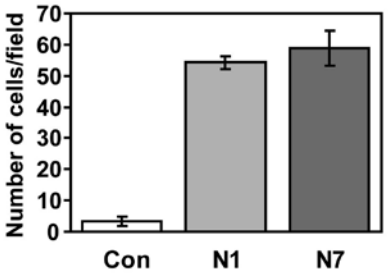

Figure 3. NHE7-overexpression or NHE1-overexpression enhances the cell invasiveness of breast cancer MDA-MB-231 cells. Equal numbers of each cell line were placed in the top chamber and the number of cells that migrated to the bottom chamber was counted after 48 h of incubation. The data presented are the mean ± SD of the number of cells counted in five randomly chosen fields. Con, control MDA231 cells; N1, NHE1 MDA-MB-231 cells; N7, NHE7 MDA-MB-231 cells.

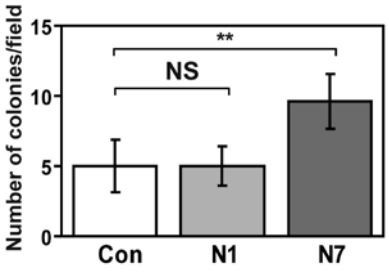

Figure 4. NHE7-overexpression facilitates colony formation on soft agar. Equal numbers of N1, N7 and c-MDA231 cells were seeded onto soft agar and colony formation was assessed after 6 weeks of incubation. The data presented are the mean ± SD of the number of colonies counted in five randomly chosen fields. A set of a representative data of three independent experiments is shown. $^{**}p<0.01$ by the Student's t-test. NS, not significant; Con, control MDA231 cells; N1, NHE1 MDA-MB-231 cells; N7, NHE7 MDA-MB-231 cells.

gate the potential involvement of NHE7 and NHE1 in tumor invasion, we set out matrigel Transwell chamber assays. After 48 h of incubation, a significantly greater number of N1 cells (54.2±1.93 cells/field) migrated to the bottom side of the membrane than c-MDA231 cells (3.4±1.35 cells/field) (Fig. 3) consistent with the previous studies. Intriguingly, N7 cells also exhibited a similar degree of enhancement in invasion capability (58.8±5.49 cells/field) as N1 cells, indicating the involvement of both NHE1 and NHE7 in this phenotype.

Anchorage-independent tumor growth is facilitated by NHE7. Anchorage-independent growth is a hallmark of tumor progression, but little is known about the involvement of NHEs in this process. In order to explore the potential involvement of NHE1 and NHE7 in anchorage-independent tumor growth, cells were cultured in soft agar media for 6 weeks and the colonies were counted. N7 cells typically formed approximately twice more colonies on soft agar than c-MDA231 cells ($p<0.01$ by the Student's t-test) whereas N1 formed similar numbers of colonies as c-MDA231 cells (Fig. 4), suggesting that elevated NHE7 expression levels supports anchorage-independent growth.

Tumor formation is facilitated by NHE7 overexpression. Having shown that NHE7 facilitates cell overlay, invasion and anchorage-independent tumor growth, we next examined whether NHE7 overexpression enhances tumor formation *in vivo*. N7 or c-MDA231 cells were subcutaneously implanted into two athymic female nude mice bilaterally on the back and tumor formation in the focal location was observed over time. The experiments were repeated and the cumulative results obtained from the two independent experiments are shown in Fig. 5A. After 80-100 days post-injection, all 8 sites of the 4 mice injected with N7 cells (NHE7-m1-m4, L or R) developed tumors ranging in volume from 278-510 mm^3. In contrast, one tumor that had arisen from cells stably expressing pcDNA3 vector (pcDNA-m1-R) had an area of ~30 mm^3 and the rest of the tumors (pcDNA) were barely visible by the end point of the experiments. Note that the experiments had to be terminated at the indicated time point because the tumor volume of N7 had become >500 mm^3. Pathological examination of the tumor specimens revealed that the edges of control tumors were smooth and encapsulated (red dotted line) whereas tumor cells at the edge of NHE7 overexpressing tumors invaded the host stroma as small clusters and single cells (Fig. 5B). Notably, the latter were often lined up in single file (arrows), a characteristic of infiltrating lobular breast tumors, suggesting that subcutaneous tumors derived from N7 cells may exhibit a more invasive appearance than tumors derived from c-MDA231 cells.

Discussion

We previously showed that cell adhesion molecules, cytoskeletal proteins and signaling molecules that have been implicated in tumor progression participate in the NHE7-interactome (32). It was also noted that MDA-MB-231 cells stably expressing NHE7 appeared to pile up more promptly than parental cells, a similar phenotype as anchorage-independent growth that is often observed in aggressive tumors. The objective of the present study was therefore to explore the potential impact of elevated NHE7 expression in MDA-MB-231 cells on different steps of tumor progression. Here we show that NHE7 overexpression facilitates cell-overlay and cell-cell adhesion. Recent studies began to suggest that cell adhesion molecules and cell-surface receptors control contact inhibition either positively or negatively depending on the cellular or extracellular contexts (48), and cell adhesion is indeed required for certain types of tumor invasion and migration during metastasis (49-53). Interestingly, we have shown in this study that NHE7-overexpression facilitates tumor invasion, cell-overlay and tumor formation *in vivo*. NHE1 is known to facilitate tumor invasion by acidifying the local pH and thereby activating pH-sensitive enzymes that degrade the extracellular matrix (9,40-45). In agreement, we also showed that overexpression of NHE1 in MDA-MB-231 cells enhances cell invasion (N1 in Fig. 3), but does not affect cell adhesion nor anchorage-independent growth. Thus, NHE7 and NHE1 may control tumor progression by acting on a common pathway, while distinct targets may also exist that are preferentially regulated by NHE7. One of the mice injected with N7 cells (mouse NHE7-m1, Fig. 5A) developed two secondary subcutaneous tumors at a distance from the primary site (data not shown). Although we have to be cautious about the possibility that it

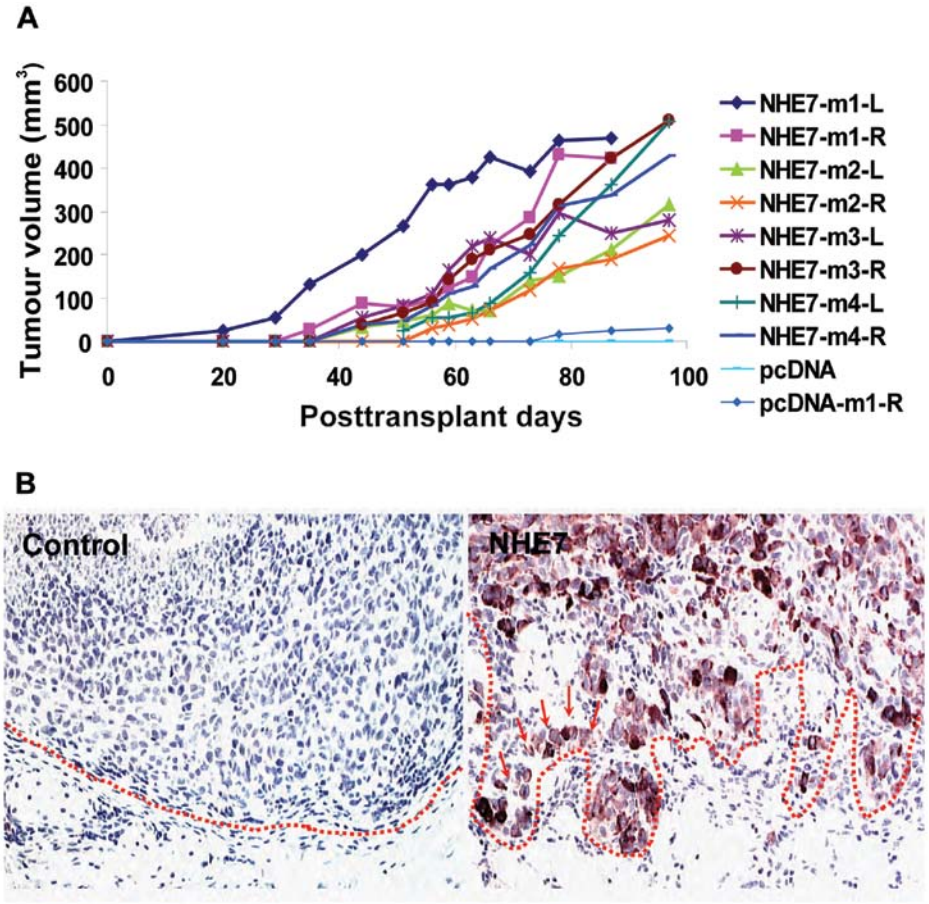

Figure 5. (A) NHE7-overexpression facilitates tumor formation *in vivo*. Clonal MDA-MB-231 cells stably expressing 1D4-tagged NHE7 or the empty pCDNA3 vector ($4x10^6$) were subcutaneously implanted into 5-6-weeks-old female athymic nude mice and the size of the primary tumor was measured. Four mice were bilaterally injected with NHE7 on the back (NHE7-m1, m2, m3 and m4) and another 4 mice were similarly injected with vector expressing cells (pcDNA-m1, m2, m3 and m4). L and R indicate the tumors implanted on the left side and right side of the back, respectively. Data obtained from two experiments are cumulatively expressed in the figure (m1 and m2 are obtained from the first experiment, m3 and m4 are from the second experiment). Note that the experiments were terminated when the tumor size exceeded 500 mm^3. (B) NHE7-overexpression increases the invasiveness of breast tumor cells *in vivo*: Control MDA-MB-231 cells (left panel) and cells stably overexpressing 1D4-tagged NHE7 (right panel) were grown as sub-cutaneous xenografts. Paraffin sections of the primary tumors derived from control MDA-MB-231 cells (pcDNA) and NHE7-overexpressing cells (NHE7-m1-R) were immunostained with an anti-1D4 mouse monoclonal antibody to visualize NHE7. Note that the edge of the control tumors was smooth and encapsulated (red dotted line) while tumor cells at the edge of NHE7 overexpressing tumors invaded the host stroma as small clusters and single cells. The latter were often lined up in single file (arrows), a characteristic of infiltrating lobular breast tumors.

was caused by host problems, such as an anatomical abnormality (e.g., diverged lymph or blood vessels), it is tempting to postulate that NHE7 may mediate tumor spreading and possibly metastasis.

The acidic pH of the organellar lumen along the secretory (54,55), endocytic (56-58) and recycling pathways (59,60) is a key determinant of vesicular trafficking. Consistent with the notion that organellar NHEs regulate organellar pH, deletion of NHX1 in yeast *S. cerevisiae* (18,61) and gene suppression of human NHE6 (62) and NHE8 (63) impaired distinct vesicular trafficking pathways. Thus, it is likely that NHE7 also regulates vesicular trafficking. Regulation of vesicular trafficking, particularly the balance between endocytotic and exocytotic (secretion) membrane dynamics, has attracted increasing amounts of attention as a mechanism of tumor invasion (64-68). During tumor invasion, a constant unidirectional membrane flow towards the leading edge provides phospholipids and membrane proteins with a confined area of the plasma membrane (69-71). The selective delivery of specific molecules such as cell adhesion molecules, signaling molecules, cytoskeletal proteins and scaffold proteins (72-76) to the leading edge also establishes the cell polarity in migrating cells. Thus, our current study suggests that NHE7 is a promising candidate for linking organellar homeostasis, vesicular trafficking and tumor progression.

Compartmentalized NHE-activity at the leading edge is an emerging mechanism of tumor invasion. In addition to the effect on pH, NHE activity at the leading edge also enhances local cell swelling by a water influx coupled with a Na^+ influx, which most likely provides the driving force for cell migration (77,78). Although the main function of organellar NHEs has been attributed to organellar pH and cation homeostasis (12,13,16,20), organellar NHEs can be targeted to a specialized compartment of the cell-surface membrane and may show functionality depending on the cellular/extracellular contexts. For example, a small population of NHE6 and NHE9 is delivered to the hair bundle, a specialized structure of the apical side of the cell, and the potassium highly enriched in the endolymph drives the H^+/K^+ exchanger across the hair bundle (79). An interesting

finding is that a small but notable population of NHE7 (~2%) is targeted to the cell surface (33,80), and that NHE7 is associated with caveolin-positive lipid rafts (membrane compartments enriched with cholesterol and sphingolipids) (32,80) and the focal complex (32). Moreover, CD44 binds to NHE7 most likely in the lipid raft fraction (32). As CD44 binds to and activates NHE1 in the caveolin-enriched membranes of the leading edge of migrating MDA-MB-231 cells (40), it is tempting to speculate that NHE7, CD44 and caveolins may be associated in a close proximity to NHE1 at the leading edge of invasive tumors (9). Despite all attempts, we were not able to detect any biochemical interactions between NHE7 and NHE1 (32), nor were we able to see any appreciable effects of NHE7-overexpression on the overall cytosolic pH (16). This is not surprising in light of the temporally-regulated and the spatially-confined NHE7 expression on the cell surface. It will be important in the future to develop a sensitive pH probe that can be targeted to confined membrane microdomains such as the leading edge of migrating cells to define the local pH in live cells.

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